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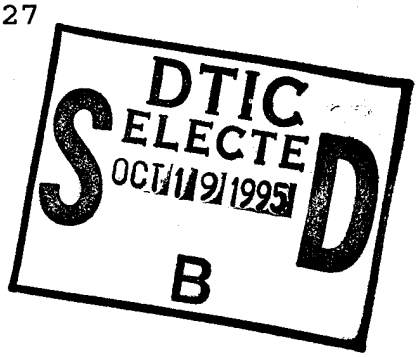

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Mutation of the p53 gene is very frequent in breast cancer. In normal cells induction of wild-type p53 function leads to either cell cycle arrest or cell death. Loss of this function can contribute to oncogenic cell transformation. Additionally the presence of mutant forms of p53 in breast tumor cells may actually facilitate the process of tumorigenesis. The properties of mutant p53 proteins in vitro and in breast tumor cell lines will be studied, Experiments will focus on analysis of the structure and modification of mutant p53 proteins as well as the effect of cellular signalling on p53 function. Additionally it is planned to establish breast cell lines expressing inducible mutant p53 to determine the effect of such mutants on parameters of cell cycle, growth and death.

We have discovered that all mutant forms of p53 tested are capable of binding specifically to p53 response elements present in p53 target genes at lower but not at physiological temperatures. Furthermore we have identified a means by which such binding is stabilized at the higher temperature. This will allow us to explore means to develop molecules that might have the outcome of converting p53 in breast tumor cells from mutant to wild-type in function. Such reagents would have clear therapeutic advantages.

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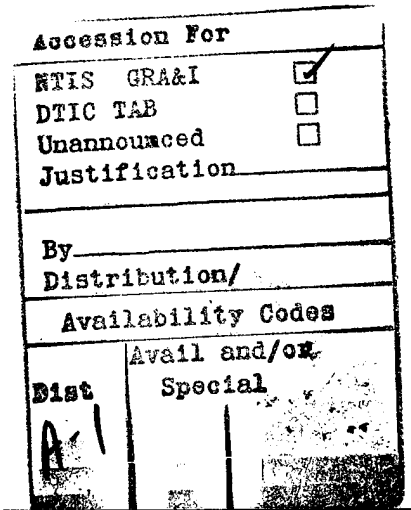

Carol Powers 8/16/95
PI - Signature Date

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The over-all goal of research supported by DAMD 17-94-J-4275 is to characterize the role of mutant p53 in breast cancer progression and to develop means to counteract the tumorigenic potential of mutant p53.

Background:

The p53 tumor suppressor protein plays a pivotal role in transmitting a signal from agents that induce genotoxic stress to genes that control the cell-cycle and apoptosis (1,2). p53 is a DNA binding dependent transcriptional activator which binds specifically to sites in genomic DNA that contain two or more copies of the consensus sequence: 5' R R R C A/T T/A G Y Y Y 3' (3). Such sites are identified as p53 response elements in a number of genes. Thus when DNA is damaged the p53 protein normally present in low quantities in cells and in a latent, inert form, is activated both quantitatively and qualitatively to induce several target genes. Among these are included the genes expressing GADD45 (4), WAF1/p21/CIP1 (5), mdm2(6), cyclin G (7), and bax (8). Each of these genes contains a p53 response element and is therefore a likely target for p53 as a transcriptional activator to induce their expression. Each thus is likely to play a role in the p53 pathway in which, as a result of DNA damage, normal cells either undergo cell cycle arrest or cell death. When p53 is mutated and cannot respond thus to DNA damage, cells display the loss of growth control that is characteristic of tumorigenesis.

Mutation of the p53 tumor suppressor gene is among the most frequent events in breast cancer. Such mutation is frequently manifested as loss of one allele coupled with missense mutation of the other allele. Strikingly the location of the missense mutations are within the central region of the molecule (9) and this region contains the DNA binding domain (10) (See Figure 1). This highlights the likelihood that specific DNA binding is essential for the tumor suppressor function of p53 and that DNA binding is absent from mutant forms of p53. The missense mutant p53 proteins are frequently expressed at very high levels in breast tumor cells (11), and the p53 status in breast cancer has been linked closely to detection of p53 protein by immunostaining (12). Therefore understanding the function of the wild-type p53 protein and how it is altered when p53 is mutated will be critical to evaluating the prognosis of breast cancer. Importantly, the study of the properties of mutant p53 in breast cancer will hopefully lead to the development of ways to convert mutant protein to wild-type in function. The original aims of this proposal are as follows:

Specific Aims:

(1) Analysis of the structural properties of mutant forms of p53 that are found in breast cancer and how they differ from that of the wild-type form as well as among themselves.

(2) Analysis of the DNA binding properties of mutant forms of p53 in breast cell lines with the aim of (a) finding cellular genes that are targets of mutant p53 activation and (b) conversion to or stabilization of the wild-type DNA binding activity of mutant forms of p53.

(3) Identification of cellular proteins from mammary cell lines that might be involved in mutant p53 gain-of-function in breast cancer.

PROGRESS REPORT

Temperature sensitive specific DNA binding by mutant p53 proteins

The missense mutations in p53 that occur with high frequency in human cancers are located within the region of the protein that spans the sequence specific DNA binding domain. We have examined in detail the DNA binding properties of several immunopurified tumor-derived mutant p53 proteins (val143→ala, arg175→his, arg248→trp, arg249 →ser and arg273→his). All but one of these (ala143) represent mutational hot-spots in p53. Our results can be summarized as follows:

(1) While all mutants were defective for binding to DNA at 37°C, by electrophoretic mobility shift assays (EMSA) each bound specifically to several cognate p53 binding sites at sub-physiological temperatures (25-33°C) (Figure 2). Our results show that both wild-type and mutant forms of p53 are temperature sensitive for DNA binding. Binding by mutant p53 proteins occurs to a lesser extent than observed with the wild-type protein, and varies somewhat with the mutation and the version of the consensus site used.

(2) Heating mutant p53 proteins at 37°C irreversibly destroyed their ability to subsequently bind at 25°C. The time course of heating also varied with the mutant, although all mutants have lost the ability to bind to DNA within seven minutes of heating at 37°C. (Figure 3). By contrast, wild-type p53 loss of DNA binding is much slower such that within 10 minutes binding is only slightly diminished and by 30 minutes of incubation at 37°C 30-40% of the original amount of DNA binding is retained (Compare Figures 2 and 3).

(3) Loss of binding occurs gradually over the temperature range examined with the greatest loss occurring between 33 °C and 37 °C (Figure 4).

(5) DNA binding by mutant p53 at lower temperatures is sequence specific as evidenced by competition EMSA (Figure 5) as well as by DNaseI footprinting and filter binding assays.

Conclusion:

It is highly likely that sequence specific DNA binding and activation of target genes is central to the function of wild-type p53. Indeed, there are a number of studies that suggest that p53 induced cell death is lost when wild-type p53 function is lost. Since the vast majority of tumor derived mutations are located within the DNA binding domain of p53 this suggests that tumor derived mutants are defective for binding to DNA. Indeed this is what we and others have found (13-19). However our discovery that all mutant forms of p53 are inherently capable of binding specifically to DNA is exciting. It opens the possibility that there may be ways to convert mutant protein to wild-type in function, with the ensuing of either arrest or (more likely) death of mutant p53-bearing breast tumors. We believe that we have made progress towards this goal.

Stabilization of mutant p53 temperature binding by antibodies that recognize a region within the p53 N-terminus

We originally identified an antibody PAb 1801 that, when bound to mutant p53 proteins stabilizes their binding to DNA at physiological temperature (37°C). This was mentioned in the original grant proposal. We have expanded this observation in the following ways:
(1) We now know that the epitope recognized by PAb 1801 is within amino acids 46-55 (20), a considerably smaller region than was estimated at the time of writing this proposal. This allows for more detailed and effective analysis of this region and its role in regulating p53 DNA binding.
(2) In collaboration with T. Soussi we have test several different monoclonal antibodies that each share the ability to recognize an epitope encompassing amino acids 46-55. We have found that these markedly stabilize binding by all mutant p53 proteins tested at 37°C (Figure 6). By contrast, antibodies that recognize epitopes located elsewhere within p53 stabilize mutant p53 binding significantly less effectively. (Figure 6).

(3) Both intact PAb 1801 antibody and FAb fragments of PAb 1801 allowed mutant p53 to bind to DNA. We estimate that for maximal binding stabilization four FAb fragments per p53 molecule are required. Thus since p53 is a tetramer each p53 monomer is required for the stabilization.

Conclusion: Our data show that the major hot-spot p53 mutants have the intrinsic ability to bind to DNA and that a unique region within the N-terminus of p53 may be critical for rescuing them from loss of binding at physiological temperatures. This suggests the possibility of developing small molecules that can stabilize mutant p53 proteins under physiological conditions.

Planned research:

(1) Examination of other modifiers of p53 function:

A major part of our research program is to understand the regulation of the wild-type p53 protein and how this regulation affects mutant versions of p53. Our's and others' research has led to the observation that the central sequence specific DNA binding region of p53 is regulated by signals and sequences outside of this domain (21-24). In particular the C-terminus of p53 is clearly capable of transmitting positive and negative signals to the DNA binding domain. The C-terminus itself contains an autonomous domain that is capable of recognizing and reannealling short complementary single strands of DNA or RNA (25-27). Based on that and other observations we have shown that short but not long single strands can stimulate the ability of the central specific binding domain to recognize and protect a cognate p53 binding site from DNaseI digestion (24). Since the p53 pathway in cells is clearly necessary for tumor suppression, and since we have shown that mutant forms of p53 can bind to DNA at lower temperatures it will be of interest to determine whether at lower temperatures mutant forms of p53 can receive signals from single strands of DNA.

We and others have also observed that manipulation of the C-terminal 30 amino acids of p53 increases sequence specific DNA binding (22,23). Indeed, it has also been shown that tumor derived mutant forms of p53 can be stimulated by antibodies which react with the C-terminus and with peptides that span the highly basic region a the C-terminus. However, our new results show that this stimulation

is greatly reduced when DNA binding assays are performed at physiological temperature (37°C). We have preliminary evidence for a cooperative effect of on mutant p53 binding by antibodies interacting with the N- and C-termini of p53. We will test several C-terminal peptides in combination with N-terminal specific antibodies or, when isolated, N-terminal peptides in order to increase the effectiveness of stabilization of mutant p53 proteins in the wild-type conformation.

Another means by which the C-terminus of p53 regulates the central DNA binding domain is thorough phosphorylation of specific residues. Phosphorylation of the cyclin dependent kinase (cdk) site at residue 315 (23), the CKII site at 392 (21) or a PKA site within residues 376-379 (28) have been shown to increase specific DNA binding by wild-type p53. We have also determined that phosphorylation of wild-type p53 by cyclin dependent kinases can alter binding site preference by p53, suggesting a novel means of regulation. It will be of considerable interest to determine whether and how phosphorylation affects mutant forms of p53.

(2) Search for DNA sequences bound by mutant p53 proteins:

We have initiated experiments to identify sequences bound preferentially by mutant forms of p53. Our initial experiments show that the "SELEX" approach for identifying p53 binding sites using tagged oligonucleotides containing random sequences as previously described by Funk et al (29) works well for our source of purified wild-type p53. It is planned to extend this approach to defining the sites preferentially bound by each hot-spot mutant form of p53 at 25°C. It is also possible that mutants are capable of binding to different sets of sequences at physiological temperature. Once experiments testing mutant p53 protein binding at 25°C are completed we will test whether any specificity for DNA binding can be demonstrated at 37°C. As an alternative approach we will use the "catch-linker" method that Vogelstein and colleagues (30) developed to identify p53 binding sites in human genomic DNA. These experiments will complement our other investigations on mutant p53 DNA binding described above.

(3) Search for cellular proteins and peptides that bind to p53:

(i) As described in the original proposal we will construct columns containing wild-type or mutant forms of p53 and attempt to affinity purify proteins that interact specifically with each form. Our observation of the temperature sensitivity of p53 is important for this goal. Initially when we attempted this we were unable to detect significant differences when experiments were carried out at 4 °C. With our increased understanding of the effect of thermal stress on wild-type and mutant forms of p53 we will repeat these experiments using several mutant forms of p53 in comparison with wild-type protein.

(ii) Using phage display libraries (31, 31a) we will attempt to isolate peptides that bind tightly to the N-terminus or the PAb 1801 epitope (amino acids 45-46) region of p53. Such an approach has been used successfully for mapping p53 epitopes (32). If peptides can be identified by this approach we will characterize them by affinity and specificity for p53 with the ultimate goal of determining whether any are capable of stabilizing mutant p53 DNA binding and transcriptional activation in vitro or after introduction into breast cells.

(4) Transcriptional regulation

The DNA binding property of p53 is relevant to its function as a transcriptional activator. It is planned to examine the ability of wild-type and mutant p53 to activate transcription in vitro. We have established in vitro transcription systems for comparison of wild-type and mutant p53 activation of transcription of templates containing p53 response elements. We will determine whether the mutants are temperature sensitive for transcriptional activation, or repression. We will test the effect of different antibodies, peptide and phosphorylation by different kinases on the ability of mutant forms of p53 to regulate transcription.

(5) Mutant p53 proteins in breast cells.

Several breast tumor cell lines are available whose p53 status has been established (eg 33). They contain either wild-type p53, no p53 or missense mutant p53. We will test the ability of the missense mutant forms of p53 in cell extracts to bind to DNA, to examine the effects of antibodies, C-terminal peptides and phosphorylation on these mutants. If we identify smaller molecules through phage display libraries that bind to the region containing amino acids 45-56 these will be tested on p53 mutants in cell extracts, or after introduction into cells by microinjection.

(6) Establishment of inducible mutant p53 in breast cell lines

We are interested in further exploring the likelihood that mutant p53 proteins confer selective growth advantage to cells that is increased in their increased tumorigenicity (34) MB453 and MB157 are p53 null breast cancer cell lines that are currently being grown in our laboratory. Stable derivatives of these line expressing inducible hot spot mutant p53 proteins will be constructed using the tetracycline/tet repressor system (35), The ability of mutant p53 to affect growth and other parameters will be tested. We will determine colony formation on soft agar, expression of the multi-drug resistant (MDR) promoter and altered cell cycle progression, ability to undergo growth factor dependent apoptosis and radiation induced apoptosis.

SUMMARY:

There is by mow convincing that the p53 status of tumor cells affects the outcome of cancer therapy (eg). By understanding how mutant forms of p53 differ in structure and function from wild-type p53, and by developing means to affect the status of p53 in breast tumor cells it is hoped to provide real therapeutic benefit as a consequence of the proposed research funded by this grant.

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Figure Legends

Figure 1: Landmarks of p53 structural domains and signals with location of tumor derived mutations.

Figure 2. Mutant p53 proteins bind DNA at 25 °C.

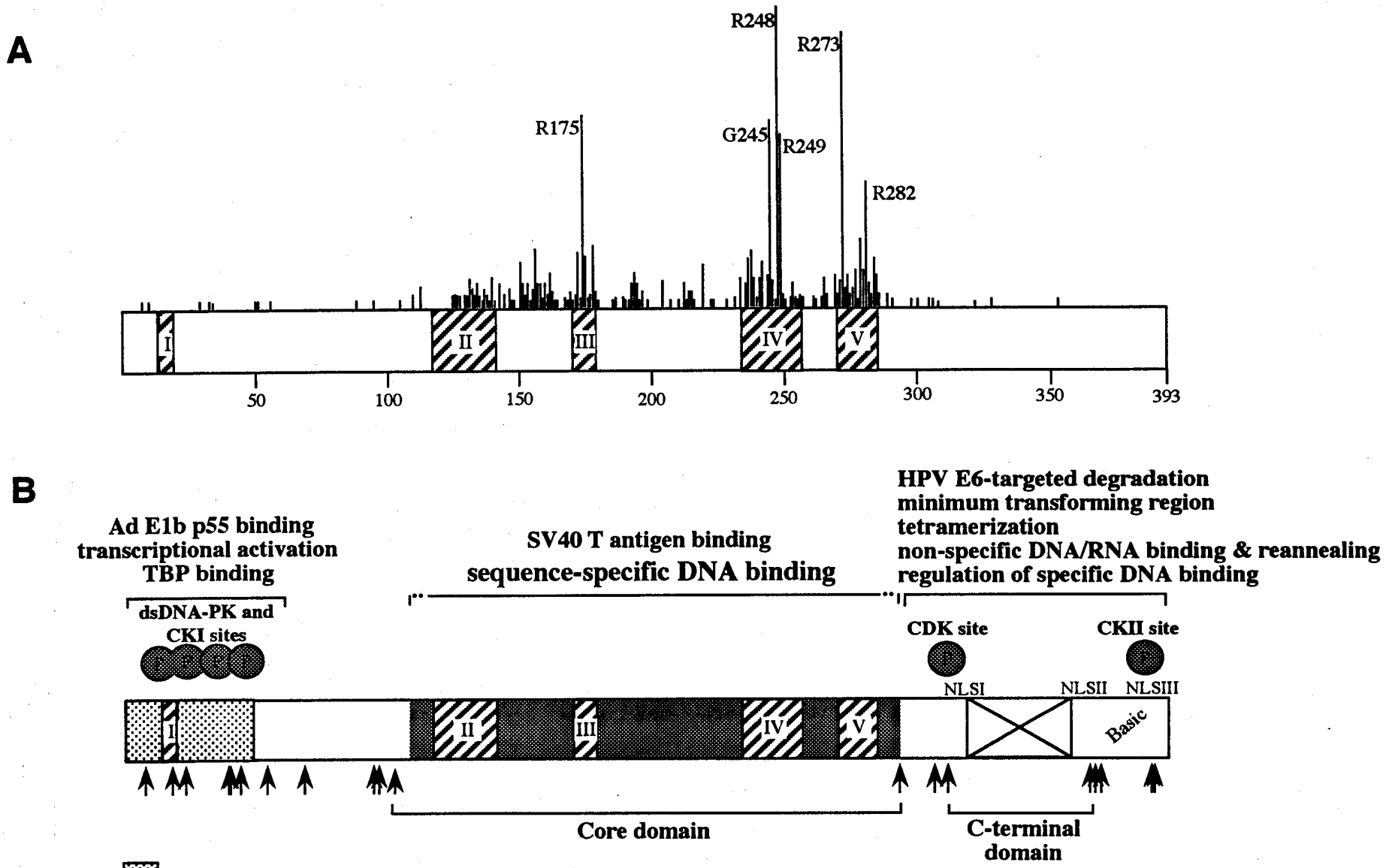
Gel mobility shift assays were used to analyze the DNA binding properties of wild-type and mutant p53. DNA binding to [^{32}P]-labeled SCS and RGC oligonucleotides (8 ng) was analyzed. Radiolabeled p53-DNA complexes were scanned and quantified using a phosphorimager. The relative levels of binding by 400 ng of p53 are shown graphically with binding by wild-type p53 at 25 °C set at 100 %.

Figure 3. Relative heat stability of wild-type and mutant p53 proteins.
Wild-type or mutant p53 proteins, as indicated, (240 ng) were preincubated at 37 °C for 0, 2, 5, 7, or 10 minutes. Then [^{32}P]-labeled RGC oligonucleotide (8 ng) was added and mixtures incubated for 30 minutes at 25 °C. Radiolabeled p53 - DNA complexes were scanned and quantified using a phosphorimager. The relative levels of binding are shown graphically with binding by wild-type or mutant p53 at 0 minutes of preincubation set at 100%.

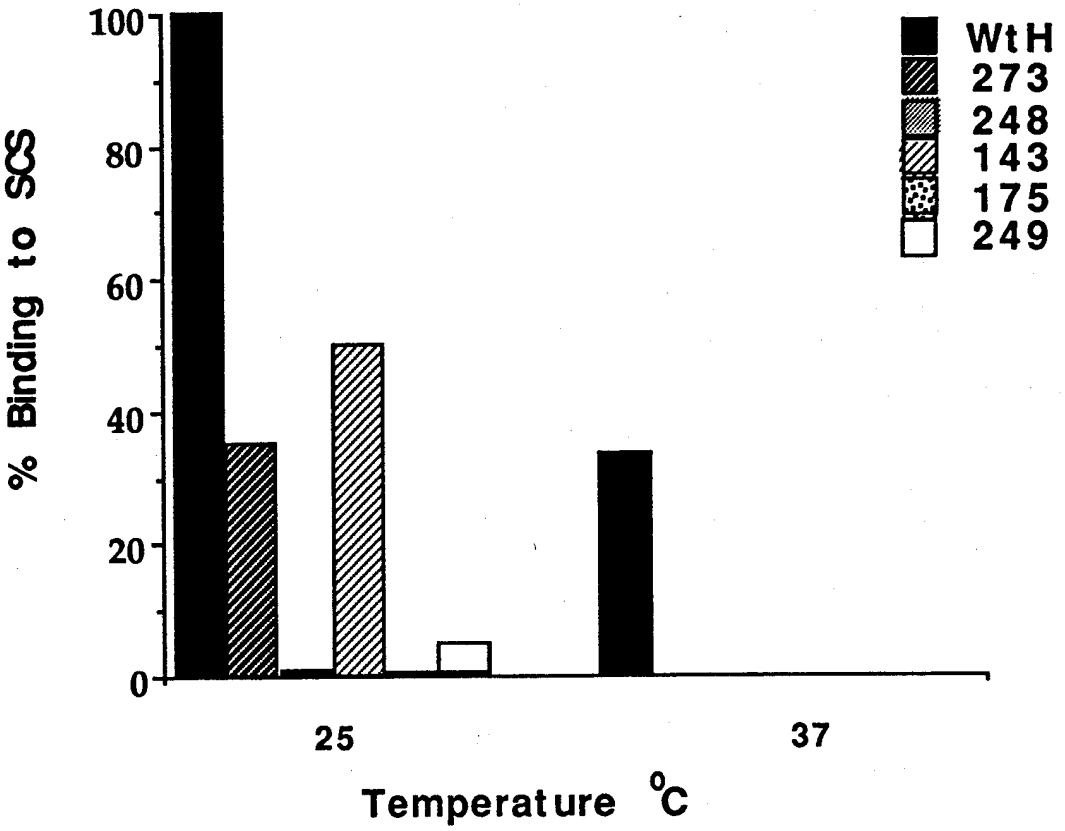
Figure 4. Temperature-dependent inactivation of mutant p53 DNA binding.
Wild-type or mutant p53 proteins as indicated (600 ng) were incubated with [^{32}P]-labeled RGC oligonucleotide (8 ng) at 25 °C, 30 °C, 33 °C, and 37 °C for 30 minutes. Radiolabeled p53-DNA complexes were scanned and quantified using a phosphorimager. The relative levels of binding are shown graphically with binding to RGC at 25 °C set at 100 %.

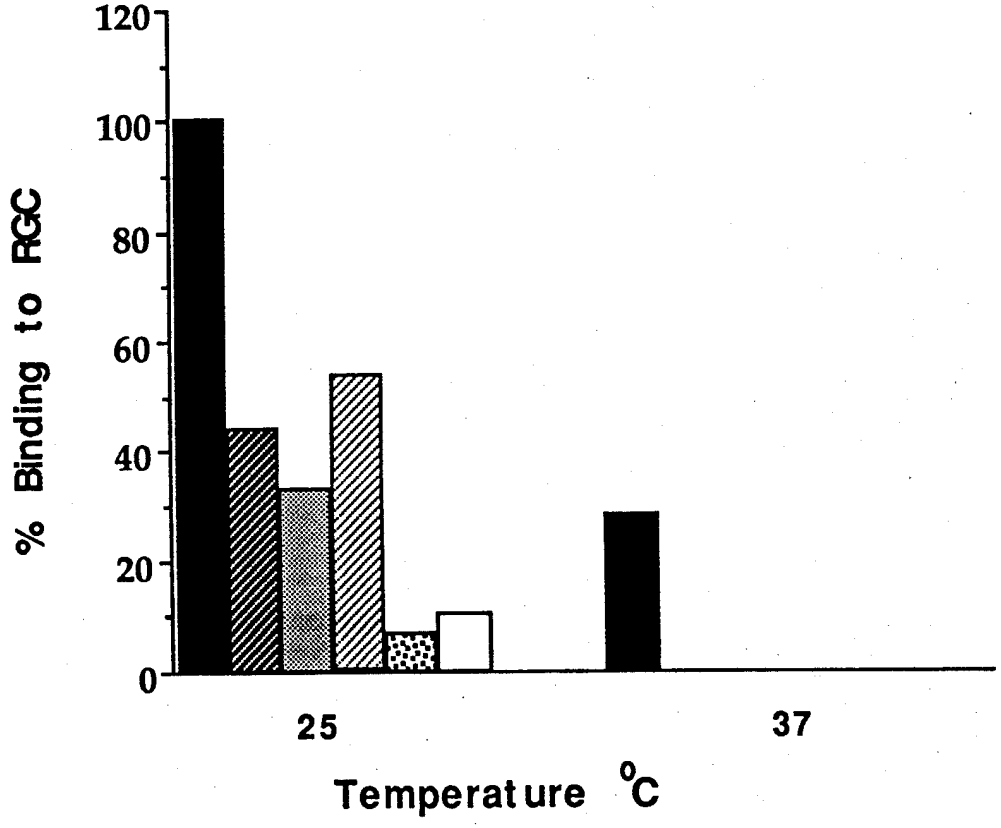
Figure 5. Sequence specific DNA binding by mutant p53 proteins.
a. Wild-type or mutant p53 proteins as indicated (200 ng) were bound to [^{32}P]-labeled oligonucleotides in the presence of unlabeled oligonucleotides as indicated. Mixtures with wild-type p53, p53(ala143) and p53(his273) contained [^{32}P] RGC oligonucleotide as probe, while mixtures with p53(ser249) or p53(trp248) contained [^{32}P] GADD45 oligonucleotide as probe. Electromobility shift assays were performed and the DNA-protein complexes quantitated using a phosphorimager. Binding with no competitor was set at 100 %.

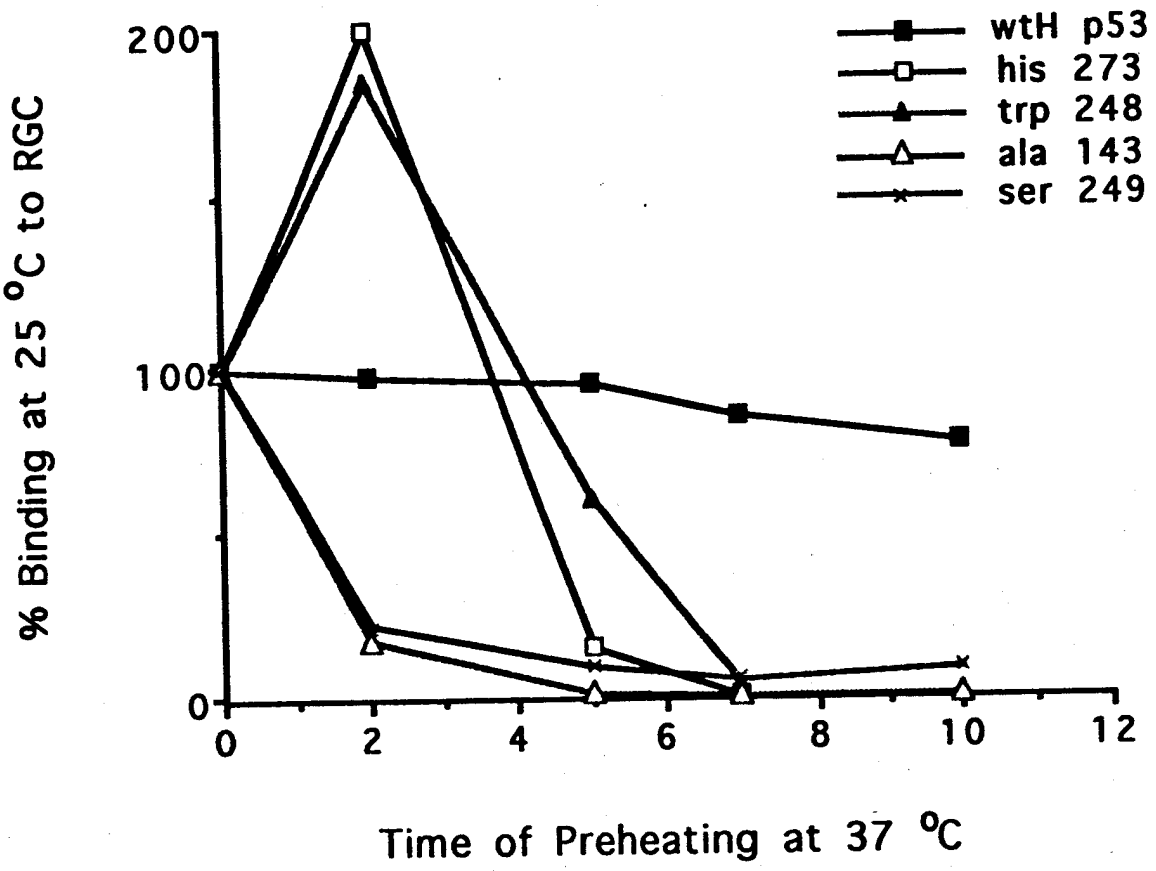
Figure 6. Survey of antibodies affecting mutant p53 DNA binding at 37 °C
Gel mobility shift assays were performed to determine the relative ability of various antibodies to stabilize binding by p53(ala143) (a) and p53(his273) proteins to [^{32}P]-labeled GADD45 oligonucleotide (8 ng) at 37 °C. 200 ng of p53 proteins were used. Binding was determined over a range of antibody concentrations (up to 750 ng) and the maximal protein-DNA complexes obtained were quantitated using a phosphorimager. We used the values obtained for plateau levels of binding. Maximal binding in the presence of PAb 1801 was set at 100 % and the ability of the various antibodies to stabilize DNA binding were plotted relative to that value. The antibodies included: L134 (epitope: amino acids 11 - 20) as ascitic fluid from mouse diluted 10 fold in PBS; B17, C36 and H461 (epitope: amino acids 16 - 30), PAb 1801, H279, and H447 (epitope: amino acids 46 - 55), HP64 (epitope: amino acids 171 - 185), PAb 421 (epitope: amino acids 370 - 378) and HR 231 (amino acids 371 - 380) all in PBS; and X77 (epitope: amino acids 16 - 25) in DMEM + 10 % FCS.

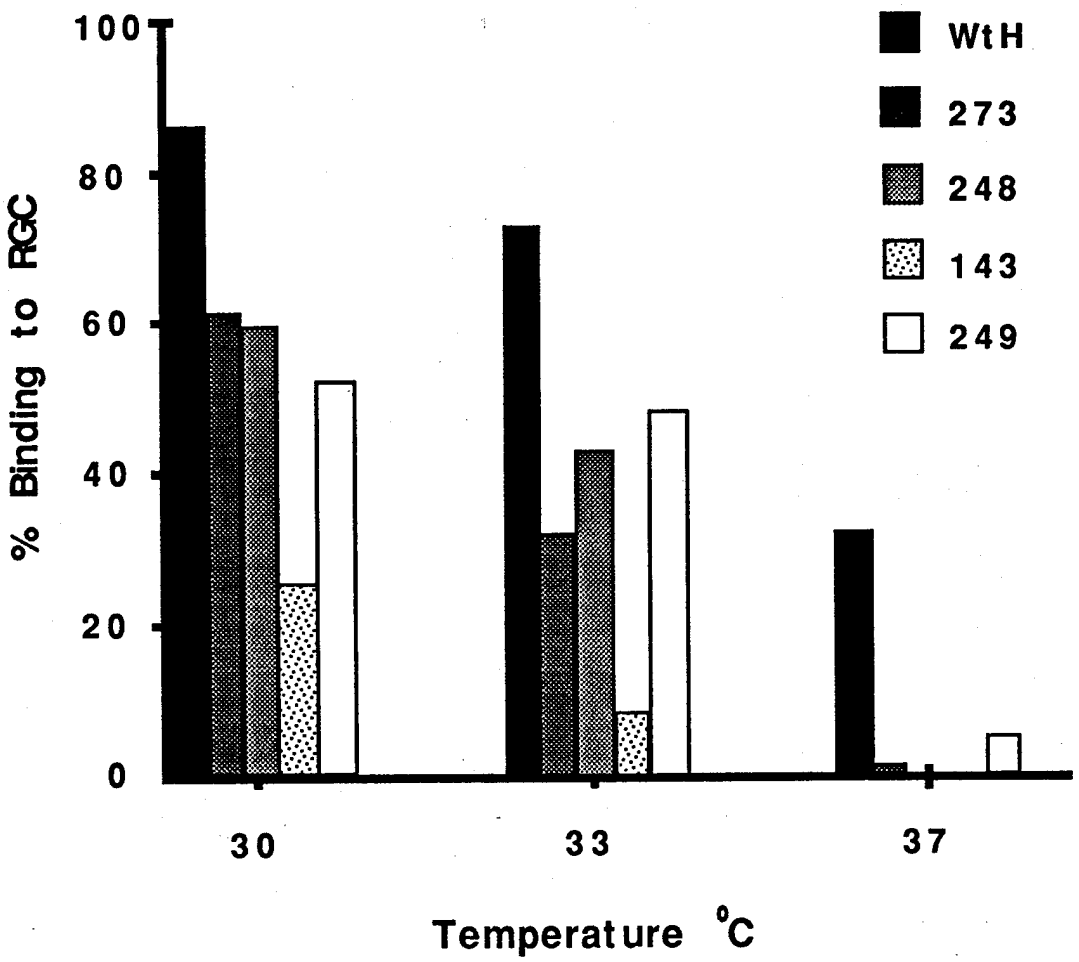

A
R248
R273
R175
G245
R249
R282
I
II
III
IV
V
50
100
150
200
250
300
350
393
B
Ad E1b p55 binding
transcriptional activation
TBP binding
SV40 T antigen binding
sequence-specific DNA binding
HPV E6-targeted degradation
minimum transforming region
tetramerization
non-specific DNA/RNA binding & reannealing
regulation of specific DNA binding
dsDNA-PK and
CKI sites
CDK site
CKII site
NLSI
NLSII
NLSIII
Basic
Core domain
C-terminal
domain
Activation
Sequence-specific DNA binding
Tetramerization
Subtilisin cutting site

c.

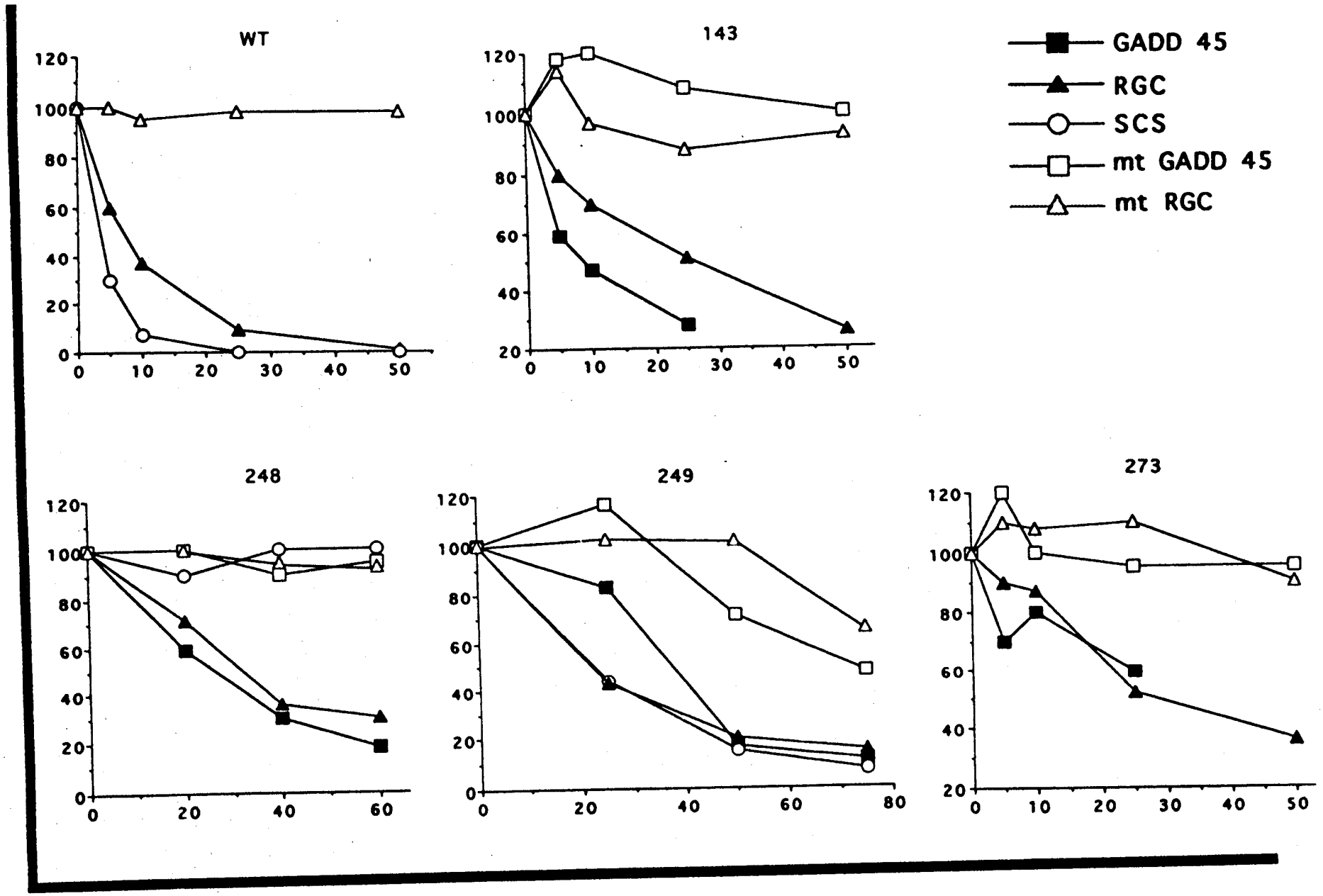

100
80
60
40
20
0
% Binding to SCS
WtH
273
248
143
175
249
25
37
Temperature °C


120
100
80
60
40
20
0
% Binding to RGC
25
37
Temperature °C

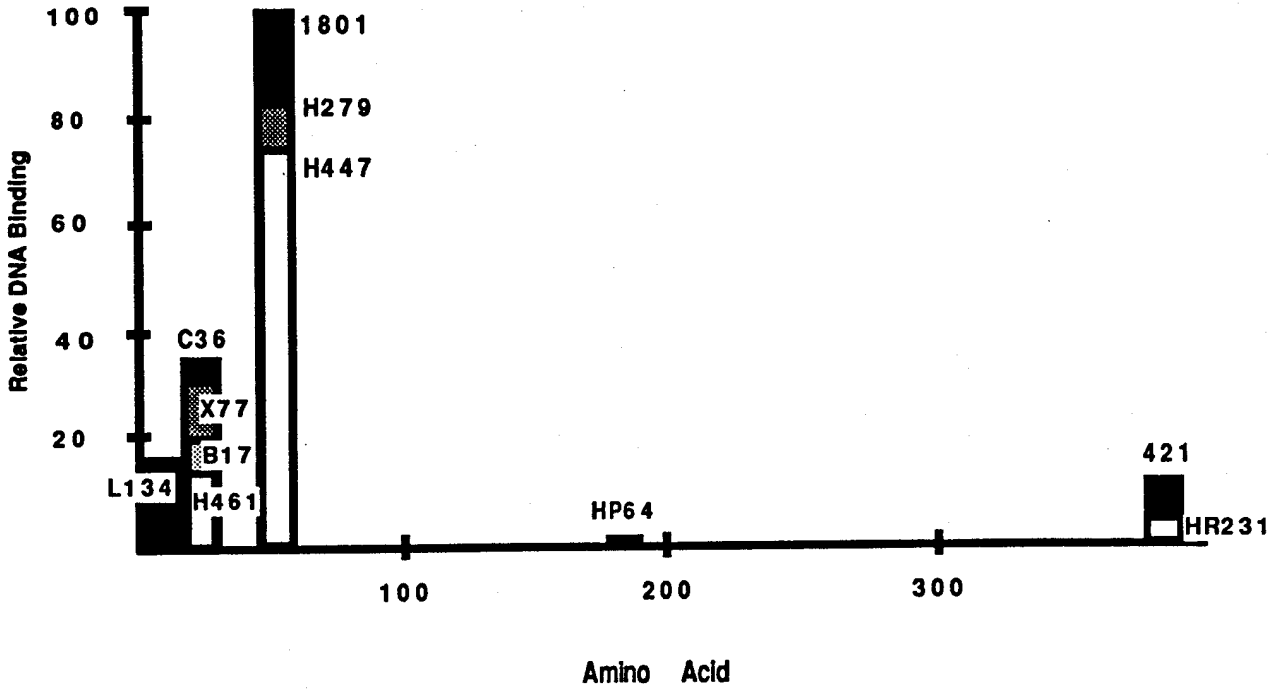

wtH p53
his 273
trp 248
ala 143
ser 249
200
100
0
0
2
4
6
8
10
12
% Binding at 25 °C to RGC
Time of Preheating at 37 °C


100
80
60
40
20
0
% Binding to RGC
WtH
273
248
143
249
30
33
37
Temperature °C

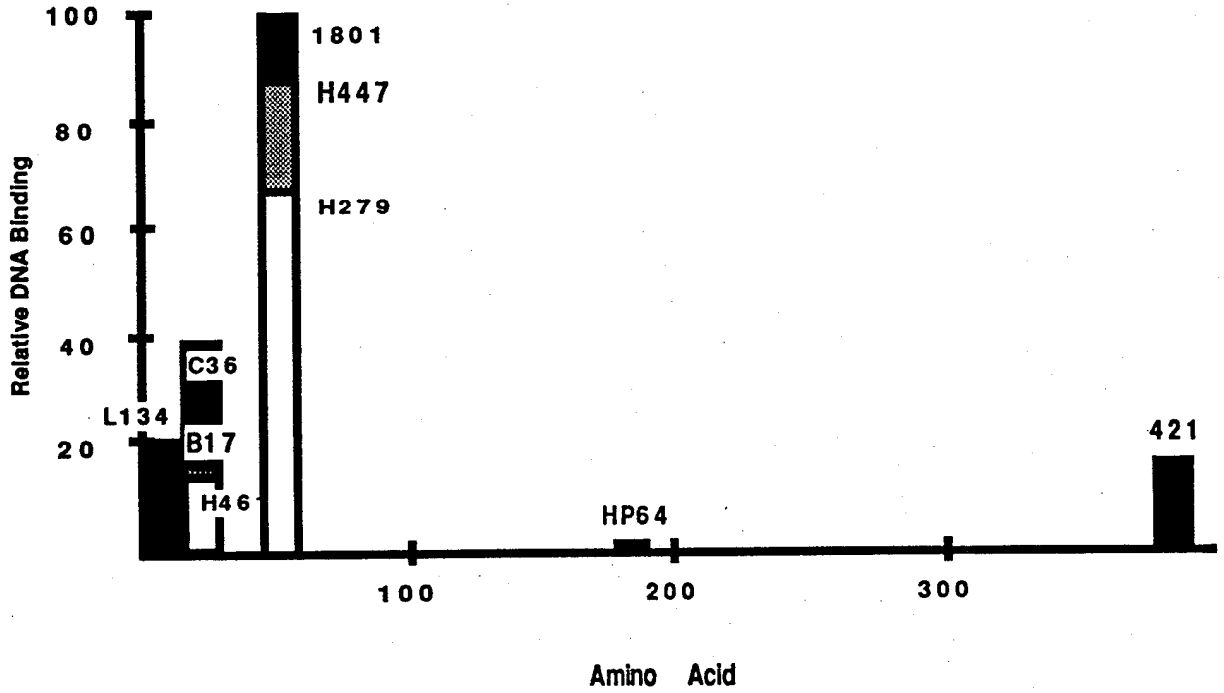
b.


WT
143
248
249
273
GADD 45
RGC
SCS
mt GADD 45
mt RGC
% Binding
Fold Excess Competitor

a.


100
80
60
40
20
Relative DNA Binding
1801
H279
H447
C36
X77
B17
L134
H461
HP64
421
HR231
100
200
300
Amino Acid

b.


100
80
60
40
20
Relative DNA Binding
1801
H447
H279
C36
L134
B17
H46
HP64
421
100
200
300
Amino Acid